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Article Addendum

Organic vs inorganic

What makes the major contribution to osmotic adjustment in bacteria?

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In order to survive hyperosmotic stress bacteria should adjust their cell turgor to altered conditions by increasing the intracellular osmolality. The classical view is that bacterial osmotic adjustment is achieved via accumulation of so-called "compatible solutes"—some organic osmolytes that can be accumulated in the cytosol at high concentrations without interfering with cell metabolism. In our recently published paper,[11] we have shown that in the absence of osmolytes in the environment uptake of inorganic ions (and, specifically, K^+) is central to osmotic adjustment in *E. coli* under hyperosmotic stress conditions. Here we show that optimal *E. coli* growth, observed at 2% NaCl, corresponds to an osmotic balance between external and internal osmolality within bacterial cells. This is achieved by the regulation of net K^+ fluxes across the bacterial membrane. We suggest that the role of compatible solutes in osmotic adjustment in bacteria is indirect and confined to the fine tuning of a number of ion channels and transporters in order to achieve osmotic balance.

High concentrations of salt or sugar are often used in the food industry to control bacterial growth in food products. Unless the cytosolic osmolality is adjusted to one of the external media, the resultant water efflux from the cell may lead to the plasmolysis and eventually to cell death. To avoid this lethal scenario, bacteria should adjust their cell turgor to altered conditions by increasing the intracellular osmolality.

The classical view is that bacterial osmotic adjustment is achieved by means of so-called "compatible solutes"—some organic osmolytes which can be accumulated in the cytosol at high concentrations without interfering with cell metabolism.[1,2] Four major classes of compatible solutes are distinguished: sugars, polyoles, amino acids and quaternary amines.[1,3] Numerous reports are available suggesting that concentrations of these compatible solutes may increase many-fold under hyperosmotic stress conditions (reviewed in refs. 1 and 4), with this increase being interpreted as required for water retention within the cell. Being first established in mid-60s, this view still dominates the literature[3,5] and has become almost a paradigm.

However, quite often (if not always) these conclusions are not substantiated by the actual numbers. *E. coli* can grow at NaCl concentrations up to 8%.[6,7] Bacterial growth has been observed in various foods containing high NaCl concentrations (e.g., 7% NaCl—as in brine of Feta cheese, reviewed in ref. 8; 8%—as in salami, reviewed in ref. 9). To compensate for it, bacterial cells would require taking up or synthesizing de novo massive quantities of organic osmolytes. Patchett et al.[10] reported that increasing NaCl concentration from 0 to 7.5% has resulted in an increase of the total amino acid pool (a major classes of compatible solutes) from 166 to 716 mM. This would result in increase in osmotic potential by 1.35 MPa. At the same time, the resultant shift from 0 to 7.5% NaCl requires an increase in osmotic potential by ca 2.55 MPa. Therefore, the above increase in the total amino acid pool may be responsible for only 50% of the required osmotic adjustment. In the absence of compatible solutes in the surrounding environment their de novo synthesis is the only option. As such organic osmolytes synthesis is relatively slow and operates in a time-scale of hours,[2] some other options should be available in order to protect bacteria in the meantime.

What is it then that makes up the rest (and the major component) of the cell's osmotic potential? In our recently published paper in *Environmental Microbiology*[11] we have shown that uptake of inorganic ions (and, specifically, K^+) is central to osmotic adjustment in *E. coli* under hyperosmotic stress conditions. Interestingly, the maximum bacterial growth was observed at 2% NaCl in the growth media and corresponded to the highest K^+ content in the cell.[11] Here we show that this optimal bacterial growth corresponds to osmotic balance within bacterial cells achieved at this NaCl concentration (Table 1).

Net K^+ fluxes have been quantified using non-invasive microelectrode ion flux data (see Fig. 1 in ref. 11) using the MIFE[12] technique (Table 1). Assuming the *E. coli* cell to be a cylinder of 1 μm diameter (D) and 2.7 μm length (L), then the half-surface area (through which measured K^+ fluxes occur) is $S_{1/2} = \pi * D/2 * L = 4.24\ \mu m^2$, and the cell volume $V = \pi * D^2/4 * L = 0.125\ \mu m^3$. Assuming K^+ flux being uniform over the surface, in 40 min (time required for the transient to be completed; please refer to Fig. 1 in ref. 11) it will cause K^+ uptake (in mol) N = Flux * time = Flux * 2400. According to van't

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Table 1 **Contribution of K^+ flux to osmotic adjustment of *E. coli* cells grown under NaCl conditions**

NaCl treatment	External osmolality, MPa	K^+ flux, nmol m^2 s^{-1}	$\Delta\Psi$, MPa	Osmotic imbalance, MPa
1%	0.19	31.17 ± 3.06	0.45	-0.26
2%	0.51	28.57 ± 0.77	0.42	**0.09**
3%	0.83	-0.64 ± 3.94	-0.01	0.84
4%	1.15	-20.32 ± 3.80	-0.30	1.45
5%	1.47	-24.66 ± 4.07	-0.36	1.83
6%	1.79	-29.57 ± 4.08	-0.43	2.22
8%	2.42	-31.58 ± 2.37	-0.46	2.88
10%	3.06	-36.1 ± 5.21	-0.53	3.59

The last two columns show changes in the cell's osmotic potential, $\Delta\Psi$, caused by NaCl-induced K^+ fluxes (calculated according to van't Hoff's law), and the overall osmotic imbalance (calculated as a difference between external osmolality and $\Delta\Psi$). Average K^+ flux values over the first 20 min after stress application are shown (mean ± SE; n = 8–13). Note: it is assumed that the cell density in the monolayer is ~80%.

Hoff's law, this will change the cell osmotic potential by $\Delta\Psi = -\Delta N * RT/V$. As shown in Table 1, osmotic balance will be achieved at 2% NaCl almost *exclusively* by means of controlled K^+ transport across the cellular membrane. This corresponded to the highest intracellular K^+ content within the range of NaCl concentrations tested.[11] At high external NaCl concentrations, NaCl-induced membrane depolarization (Fig. 5A in ref. 11) results in a substantial K^+ leak, causing osmotic imbalance (Table 1). Given the lack of organic osmolytes in the external media during experiments (the latter were performed in the defined media), bacteria could fulfill the osmotic adjustment by either using Na^+, or by increasing de novo synthesis of compatible solutes. The first avenue is detrimental to cellular metabolism, due to Na^+-specific toxicity for enzymatic reactions.[13,14] The second option has the disadvantage of being energetically very expensive (between 40 and 50 mol of ATP is needed to synthesize 1 mol of proline or glycine betaine; reviewed in ref. 15). As a result, cellular metabolism is impaired and the rate of bacterial growth is reduced. In agreement with this, increase in intracellular Na^+ was shown to coincide with a decrease in K^+ content and cell growth reduction.[11] This strongly suggests that two inorganic ions, K^+ and Na^+, have made a major contribution to *E. coli* osmotic adjustment under these conditions, while the contribution of organic osmolytes was less important. Thus, not only halophytes, but also non-halophilic bacteria such as *E. coli* rely mainly on inorganic ions for the osmotic adjustment in the absence of compatible solutes in the environment.

What then is the role of compatible solutes? Is the multi-fold stress-induced elevation in the level of organic osmolytes in bacterial cells simply "physiological noise"? It should be noted in this context that it was shown recently (although on plants) that compatible solutes may indeed be involved in osmotic adjustment in plant cell, although indirectly.[16] The authors screened 26 amino acids (one of the major classes of compatible solutes) to test their ability to prevent NaCl-induced efflux from plant roots. They showed that only a few of the amino acids were efficient in doing this at physiologically relevant concentrations (K_m between 0.3 and 0.7 mM). It was suggested that stress-induced elevation in amino acid (and other compatible solutes) level might contribute to osmotic adjustment by retention of K^+ in the cell. It remains to be seen whether the same scenario is applicable to bacterial cells. We believe this might be the case and suggest that, like in plants, the role of compatible solutes in osmotic adjustment in bacteria is indirect and confined to the fine tuning of a number of ion channels and transporters in order to achieve osmotic balance. This suggestion now needs to be experimentally tested.

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